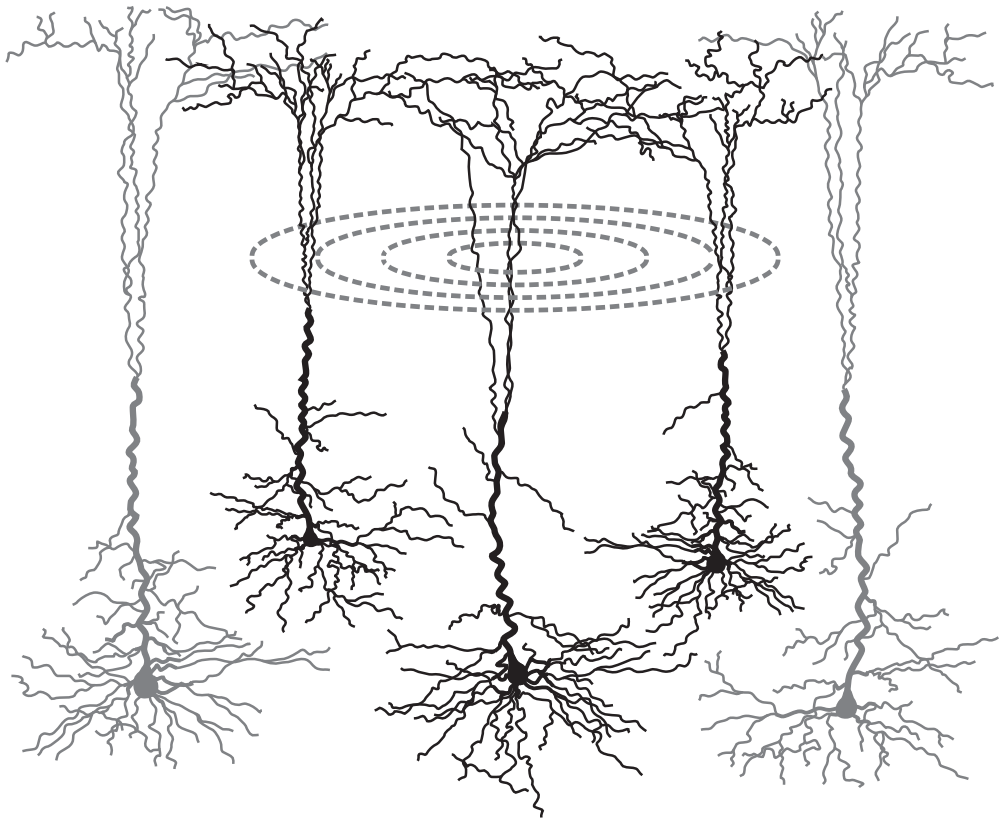
UNIVERSITY OF HELSINKI
FACULTY OF MEDICINE

NEUROPATHIC PAIN: AMYGDALOID STRESS AS CULPRIT AND CORTICAL STIMULATION AS TREATMENT

BORISS SAGALAJEV



NEUROPATHIC PAIN: AMYGDALOID STRESS AS CULPRIT AND CORTICAL STIMULATION AS TREATMENT

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To teachers, friends, and family!

CONTENTS

ABBREVIATIONS

5-HT	serotonin (5-hydroxytryptamine)
8-OH-DPAT	8-hydroxy-2-(dipropylamino)tetralin
BLA	basolateral amygdaloid nucleus
CeA	central amygdaloid nucleus
Cg	cingulate cortex
CHEM	CHEMBRIDGE-5861528
CT	central thalamus
DMH	dorsomedial hypothalamus
DRt	dorsal reticular nucleus (caudal)
GABA	γ-aminobutyric acid
GLU	glutamate
I	insular cortex
ITC	intercalated cell mass
LA	lateral amygdaloid nucleus
LC	locus coeruleus
LH	lateral hypothalamus
LIDO	lidocaine
M1	primary motor cortex
MDT	medial dorsal thalamus
mGLUR	metabotropic glutamate receptor
mPFC	medial prefrontal cortex
NMDA	N-methyl-D-aspartate
PAG	periaqueductal gray
PbN	parabrachial nucleus
PBN	phenyl-N-tert-butylnitrone
PVN	paraventricular nucleus
ROS	reactive oxygen species
RtF	reticular formation
RVM	rostral ventromedial medulla
S1	primary somatosensory cortex
S2	secondary somatosensory cortex
SC	superior colliculus
SDH	spinal dorsal horn
SNI	spared nerve injury
SNL	spinal nerve ligation
Str	striatum
t-BOOH	tert-butyl-hydroperoxide
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl

ThCN	thalamocortical network
TMS	transcranial magnetic stimulation
TRP	transient receptor potential
TRPA	ankyrin subfamily of TRP channels
TRPC	canonical subfamily of TRP channels
VLM	ventrolateral medulla (caudal)
VPT	ventral posterior thalamus
WDR	wide-dynamic range

EPIGRAPH

"It was in this way that the gray cub learned other attributes of his mother than the soft, soothing tongue. In his insistent crawling toward the light, he discovered in her a nose that with a sharp nudge administered rebuke, and later, a paw, that crushed him down or rolled him over and over with swift, calculating stroke. Thus he learned hurt; and on top of it he learned to avoid hurt, first, by not incurring the risk of it; and second, when he had incurred the risk, by dodging and by retreating. These were conscious actions, and were the results of his first generalizations upon the world. Before that he had recoiled automatically from hurt, as he had crawled automatically toward the light. After that he recoiled from hurt because he *knew* that it was hurt."

White Fang

Jack London
1906

ABSTRACT

Despite extensive research on the mechanisms of nociceptive pain, little is known about the processes that lead to neuropathic pain development. As one of the most severe and drug-resistant forms of chronic pain, neuropathic pain represents a major burden for patients, their families, and society. Therefore, new insights into the pathophysiology of neuropathic pain are needed.

The amygdala represents a complex of brain nuclei responsible for mediating negative emotions, such as fear. Moreover, maladaptive changes in the amygdala contribute to the development of chronic pain and its comorbidities, such as anxiety and depression. However, little is known about the mechanisms for amygdala-mediated pain hypersensitivity in neuropathy. We therefore investigated descending pathways engaged by the amygdala for modulation of spinal nociception in neuropathic rats.

In particular, we investigated the contribution of oxidative stress in the amygdala on the development of neuropathic pain. Oxidative stress is characterized by overproduction and poor detoxification of reactive oxygen species (ROS). ROS act as endogenous agonists of transient receptor potential (TRP) channels, which account for transduction of noxious stimulation to a nociceptive signal in peripheral nerves and for nociceptive transmission in the spinal cord. We therefore assessed how blocking TRP channels and detoxification of ROS with antioxidants in the amygdala influence pain hypersensitivity and pain affect in neuropathic rats.

Furthermore, we investigated whether electrical cortical stimulation can reverse changes associated with pronociceptive activity of the amygdala. In particular, we examined descending pathways recruited by stimulation of the secondary somatosensory cortex (S2), which has proven to be an efficacious site for attenuation of drug-resistant neuropathic pain by transcranial magnetic stimulation (TMS) in patients.

In the present series of studies, we demonstrate that N-methyl-D-aspartate (NMDA), $TRPA_1$, and $TRPC_{4/5}$ channels in the amygdala contribute to the development of neuropathic pain and that amygdaloid treatment with antioxidants can attenuate this pain. Furthermore, we provide evidence that S2 stimulation suppresses spinal nociception in neuropathic animals with hypersensitivity, but not in animals without hypersensitivity or in control animals. Medullospinal serotonergic pathways acting on the spinal 5-HT_{1A} receptor underlie the descending pain inhibition in neuropathic animals both following treatment of the amygdala with antioxidants or $TRPA_1$ antagonists and following S2 cortex stimulation.

POPULAR ABSTRACT IN FINNISH

Hermovauriokipu: mantelitumakkeen hapetusstressi aiheuttajana ja aivokuoren stimulaatio hoitokeinona

Vaikka kivun fysiologisia mekanismeja on tutkittu runsaasti, niin hermovaurion aiheuttaman neuropaattisen kivun ja kivulle herkistymisen mekanismit ovat vielä monilta osin tuntemattomia. Hermovauriokipu on usein kroonista eli pitkäkestoista ja nykyisin käytössä olevat hoitomuodot tehoavat hermovauriokipuun usein huonosti. Yleisyytensä ja vaikeahoitoisuutensa vuoksi krooninen hermovauriokipu on merkittävä hyvinvointia heikentävä tekijä yhteiskunnassa. Siksi onkin tarpeen saada lisätietoa hermovauriokivun patofysiologisista mekanismeista, joiden parempi tuntemus voi auttaa kehittämään tehokkaampia hoitomuotoja.

Mantelitumake on keskeinen aivorakenne negatiivisille emootioille kuten pelolle. Patofysiologisissa tiloissa syntyvien mantelitumakkeen rakenteellisten ja toiminnallisten muutosten tiedetään myötävaikuttavan kroonisten kiputilojen sekä niihin liittyvien emotionaalisten häiriötilojen kuten ahdistuksen ja pelon syntyyn. Mekanismit, joilla mantelitumake voi myötävaikuttaa kivulle herkistymiseen hermovauriotilanteissa, ovat vielä huonosti tunnettuja. Tässä työsarjassa tutkittiin mantelitumakkeesta lähtevän ja selkäytimeen laskevan kivun säätelyn mekanismeja kokeellisessa hermovauriokivun rottamallissa.

Erityisesti tutkimuksen kohteena oli mantelitumakkeen hapetusstressin merkitys hermovauriokivun kehittymiselle. Hapetusstressiin tyypillisesti kuuluu hapetusstressituotteiden ylituotanto sekä vähentynyt hajotus. Hapetusstressituotteet voivat tunnetusti aktivoida hermosolun kalvon TRP-ionikanavia, joiden toiminta aikaansaa ja voimistaa kipuviestejä. Tässä työsarjassa selvitettiin, kuinka mantelitumakkeeseen paikallisesti annostellut TRP-ionikanavien salpaajat tai hapetusstressituotteiden vastavaikuttajat vaikuttivat hermovamman aiheuttamaan kivulle herkistymiseen ja kivun affektiivisiin vasteeseen.

Tämän lisäksi työsarjassa selvitettiin voiko aivokuorelle annetulla sähköstimulaatiolla vaimentaa hermovauriokipua. Erityisesti tutkittiin sekundaarisen (S2) tuntoaivokuoren stimulaation vaikutusta ja mekanismeja, koska aiemmissa ihmistöissä on S2-aivokuoren transkraniaalisella magneettistimulatiolla aikaansaatu tehokas kivun lievitys potilailla, jotka ovat kärsineet hermovauriokivusta, johon tavanomainen lääkehoito on tehonnut huonosti.

Tämän työsarjan tulokset osoittavat, että mantelitumakkeen N-metyyli-D-aspartaatti reseptorit sekä $TRPA_1$ ja $TRPC_{4/5}$ ionikavat myötävaikuttavat

hermovammaan liittyvän kivulle herkistymistilan kehittymiseen. Myös mantelitumakkeen hapetusstressi, jonka synnyttämät tuotteet ovat TRP-ionikanavia aktivoivia tekijöitä, on tulosten mukaan myötävaikuttamassa kivulle herkistymiseen hermovaurion jälkeen. S2-aivokuoren sähköstimulaatio vaimensi eläinten kipuviestejä selkäydintasolla, mutta vain jos eläimellä hermovaurio ja siihen liittyvä herkistyminen kosketusärsykkeille. S2-aivokuoren sähköstimulaation samoin kuin mantelitumakkeen TRP-kanavien salpauksen ja hapetusstressin eston hermovauriokipua lievittävä vaikutus välittyi selkäydintasolle ydinjatkoksesta selkäytimeen laskevien serotonergisten solujen välityksellä, jotka selkäytimen takasarveen serotoniinia vapauttaessaan aktivoivat 5-HT_{1A} reseptoreita, jotka vaimentavat kipuviestejä selkäytimen tasolla.

LIST OF ORIGINAL PUBLICATIONS

I **Sagalajev B**, Bourbia N, Beloushko E, Wei H, Pertovaara A. Bidirectional amygdaloid control of neuropathic hypersensitivity mediated by descending serotonergic pathways acting on spinal 5-HT3 and 5-HT1A receptors. Behav Brain Res 2015;282:14-24. doi: 10.1016/j.bbr.2014.12.052.

II Wei H, **Sagalajev B***, Yüzer MA, Koivisto A, Pertovaara A. Regulation of neuropathic pain behavior by amygdaloid TRPC4/C5 channels. Neurosci Lett 2015;608:12-7. doi: 10.1016/j.neulet.2015.09.033.

III **Sagalajev B**, Viisanen H, Wei H, Pertovaara A. Descending antinociception induced by secondary somatosensory cortex stimulation in experimental neuropathy: role of the medullospinal serotonergic pathway. J Neurophysiol 2017;117(3):1200-1214. doi: 10.1152/jn.00836.2016.

IV **Sagalajev B**, Wei H, Li Z, Albayrak I, Chen Z, Tian L, Koivisto A, Pertovaara A. Oxidative stress and TRPA1 in the amygdala contribute to neuropathic pain by suppressing descending pathways acting on the 5-HT1A receptor. Submitted.

* Equal contribution

1 LITERATURE REVIEW

1.1 Introduction

Actual or potential tissue injury results from noxious stimulation and leads to acquisition of an unpleasant sensory experience, namely pain. Pain serves as an alarm system that leads to development of acute withdrawal and chronic aversive behavior for minimization of further encounter with noxious stimulation. Accordingly, pain raises our chances for a longer and healthier life. Individuals with a rare congenital insensitivity to pain usually die from severe injuries in early adulthood. This is when parents are likely to stop monitoring these individuals for tissue damage and compensating for pain malfunction [28]. At the opposite extreme are patients who experience pain from innocuous stimulation (i.e., allodynia), increased pain from noxious stimulation (i.e., hyperalgesia), or both (i.e., hypersensitivity). The lives of such patients is full of limitations and, therefore, can result in development of depression and, eventually, suicidal behavior. Although pain is subjective, it is always unpleasant. Therefore, experiences (e.g. pricking), that comprise only the sensorial aspect of pain, but fail to elicit negative emotions, are not recognized as pain by the International Association for the Study of Pain [109]. This is consistent with reports of lobotomized patients, in whom destruction of the cortex responsible for encoding of pain unpleasantness kept pain sensations from becoming bothersome [130]. At the same time, experiences, that comprise only the emotional aspect of pain [42], can be regarded as pain. In fact, such experiences can lead to development of somatic (e.g. headache) [15,53] or visceral painful sensations (e.g. chest pain) [34,37,76].

Typically, however, pain arises from activation of pain receptors, or nociceptors. Pain that results from normal somatosensory system functioning is called nociceptive, whereas pain that results from abnormal somatosensory functioning is called neuropathic. Peripheral neuropathic pain develops in the setting of peripheral nerve injury, which triggers pronociceptive changes in primary afferent fibers. In addition, pronociceptive changes can take place at the spinal and supraspinal levels and, in fact, outlast changes in the periphery [20]. In the short term, central pronociceptive changes are only functional. In the long term, these changes can lead to structural alterations [137,138]. Neuropathic pain represents the most severe and drug-resistant form of chronic pain. In the clinic, less than a half of neuropathic patients receive satisfactory pain treatment. These numbers are even lower outside the clinic. One of the main reasons for this lies in the necessity for progressive increase in the drug dosage that unavoidably leads to development of intolerable side effects. Furthermore, neuropathic pain predicts adverse

consequences for the patients' families and a substantial economic burden on society in the form of healthcare costs and patients' inability to work [119,120].

Although the processes leading to chronic pain development remain largely unknown, some of its steps are noteworthy. Patients with chronic pain display an increase in neuronal sodium channel expression [36] that likely accounts for development of hyperexcitability and spontaneous firing in nociceptors. Similarly, animals with experimental persistent pain display greater expression of cation channels, hyperexcitability of nociceptors, and activation of previously silent nociceptors [35,73]. Spinal mechanisms for facilitation of nociceptive transmission involve long-term potentiation (LTP) of synaptic strength between active nociceptive afferents and target neurons [84]. Spinal LTP requires proinflammatory activation of microglia, which may also account for a decrease in the inhibitory potency of a γ-aminobutyric acid (GABA) [26,27]. Furthermore, in the setting of nerve injury, non-nociceptive afferents can display a nociceptive phenotype [117] and, together with nociceptive afferents, sprout new branches in the spinal dorsal horn (SDH) [182] (however, see [7,63]). Altogether, this amplifies nociceptive input to the brain that, in the long-term, can lead to maladaptive changes, such as a decrease in descending inhibition and an increase in descending facilitation of ascending nociceptive transmission (for a review, see [57]).

1.2 Nociceptive pathways

1.2.1 Primary afferent fibers and their spinal targets

In primary afferent nerve fibers, conduction velocity decreases with the diameter of the fiber and thickness of myelin sheath in the following order: proprioceptive Aα, mechanosensitive Aβ, thermosensitive Aδ, thermosensitive C (unmyelinated). In addition, Aδ- and C-fibers can respond to polymodal noxious stimulation and, therefore, are responsible for nociception (for a review, see [105]). Nociceptive information from Aδ-fibers is first to arrive at the spinal cord and thus to the brain for perception of sharp and well-localized pain. Information from C-fibers arrives 1 to 2 seconds later and results in perception of burning and poorly localized pain, which increases in intensity with repetition of noxious stimulation. Furthermore, this second pain can outlast noxious stimulation and activation of C-fibers, and, therefore, likely, plays a greater role in neuropathic pain than Aδ-fibers [127].

Primary afferents project to the SDH, from where they follow separate pathways. Aδ- and C-fibers form synapses with interneurons as well as with projection neurons, axons of which cross the midline and ascend through the anterolateral column to the brain. In addition, non-nociceptive fibers of primary afferents form

synapses in the SDH, although their major branches ascend to the brain directly through the ipsilateral dorsal column. In the superficial layers of the SDH, projection neurons are mostly nociceptive specific, whereas, in deeper layers, they act as wide-dynamic-range (WDR) neurons, which converge nociceptive and non-nociceptive inputs [175].

1.2.2 Gate control theory of pain

Although reciprocal principles for encoding of nociceptive and non-nociceptive information in the spinal cord remain largely unknown, some of them appear to be consistent with the gate control theory of pain [108]. The theory rests on observations that, although both non-nociceptive and nociceptive afferents excite projection neurons, they display opposite effects on spontaneous inhibition of projection neurons by interneurons. In particular, non-nociceptive afferents directly facilitate inhibitory action of interneurons, whereas nociceptive afferents indirectly attenuate it (Fig. 1). The theory therefore suggests a circuitry, in which branches of non-nociceptive afferents can reduce ascending nociceptive transmission by "closing" the gates to projection neurons.

Interestingly, the gate control theory also indicates that, because non-nociceptive information is first to arrive at the somatosensory cortex, it likely sets receptivity of cortical neurons for the following nociceptive input and activates descending pathways for modulation of the gate control system in the spinal cord. Moreover, because the somatosensory cortex displays somatotopic arrangement, it likely triggers descending modulation capable of achieving a high level of spatial accuracy.

In addition, the gate control theory suggests that, because projection neurons receive nociceptive information both from visceral and somatic afferents (viscerosomatic convergence), the former may "open" the gates for the latter and, thus, refer the sense of pain from the viscera to elsewhere in the body. The theory therefore explains why myocardial ischemia may lead to pain development, for instance, in the left shoulder.

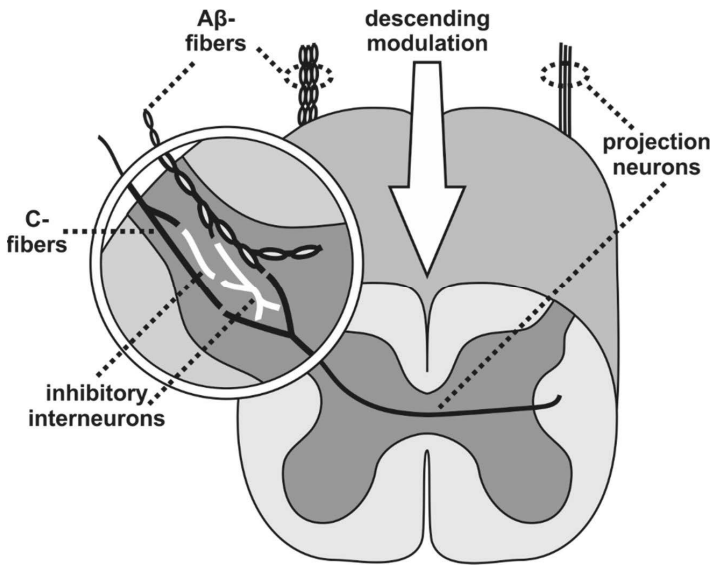

Figure 1. Gate control theory of pain. Projection neurons in the spinal dorsal horn receive main excitatory input from nociceptive C-fibers and minor excitatory input from non-nociceptive Aβ-fibers. In addition, C-fibers can excite a chain of two inhibitory interneurons and, thereby, disinhibit nociceptive transmission through projection neurons. Aβ-fibers, on the other hand, can impede such disinhibition by exciting the second inhibitory interneuron.

1.2.3 Ascending nociceptive pathways

In the brain (Fig. 2), projection neurons form the most prominent connections with the thalamus, to which they ascend directly or via the brainstem reticular formation [181]. The thalamus, in turn, relays nociceptive information to the somatosensory cortex, which encodes the sensory-discriminative aspect of pain, and to the anterior-cingulate [174] and insular cortices [21], which, in contrast, encode the affective-motivational aspect. Additional projection neurons ascend to the superior colliculus (SC), which promotes visual identification of noxious stimulation [131], to the periaqueductal gray (PAG), which acts as the opioidergic center for pain modulation [124], and to the parabrachial nucleus (PbN), which constitutes a part of the ascending arousal system [150]. Furthermore, the PbN relays nociceptive information to the hypothalamus [102,104] for regulation of endocrine responses and to the central nucleus of the amygdala (CeA) for development of avoidance behavior [12,90].

1.2.4 Descending nociceptive pathways

Medullary neurons descend to the spinal cord (Fig. 2), where they modulate nociceptive transmission from primary afferent fibers to projection neurons [56,93,151,158]. In turn, the rostral ventromedial part of the medulla (RVM) represents a target for direct modulation by higher brain structures, such as the locus coeruleus, PbN, PAG, SC, and hypothalamus [19,22,139,157,167,176]. Furthermore, indirect sources of RVM modulation can originate from the CeA, striatum (Str), primary motor cortex and likely from the S2 [4,9,10,49,59,86,87,171-173]. Such circuitry forms the neural basis for appropriate adjustment of pain threshold in a constantly changing environment. Chronic nerve injury, however, can lead to its dysregulation and, thereby, to development of pain hypersensitivity.

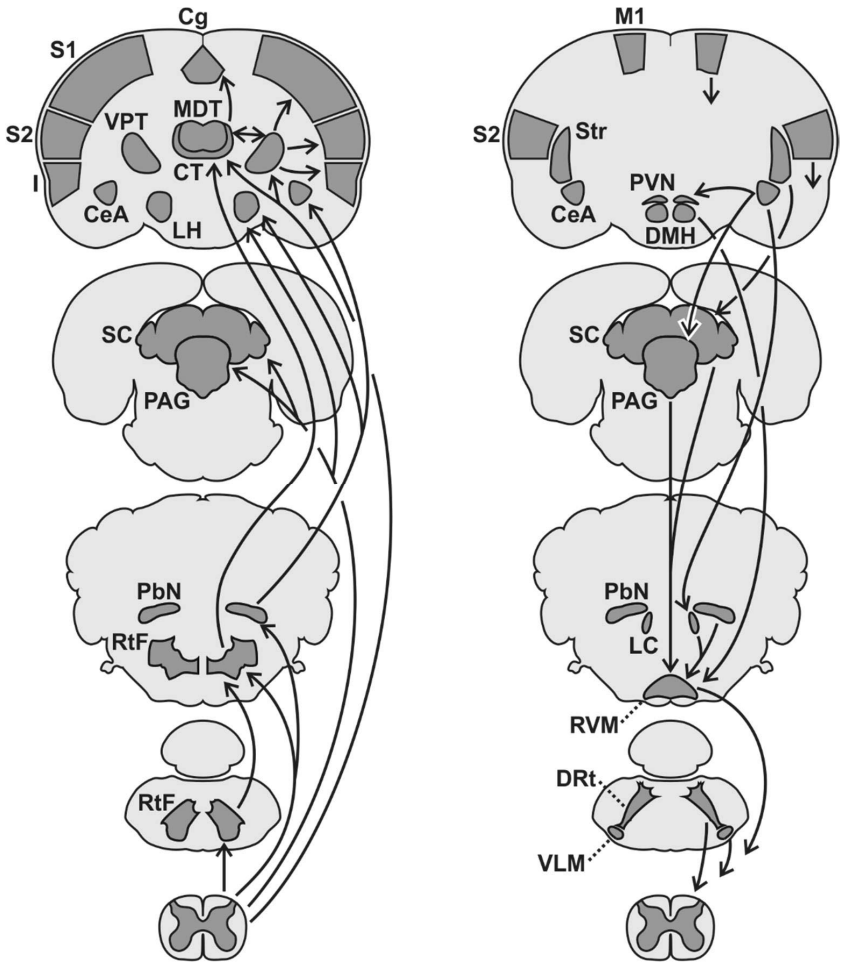

Figure 2. Ascending (left) and descending (right) nociceptive pathways.

CeA	central amygdaloid nucleus	PbN	parabrachial nucleus
Cg	cingulate cortex	PVN	paraventricular nucleus
CT	central thalamus	RtF	reticular formation
DMH	dorsomedial hypothalamus	RVM	rostral ventromedial medulla
DRt	dorsal reticular nucleus (caudal)	S1	primary somatosensory cortex
I	insular cortex	S2	secondary somatosensory cortex
LC	locus coeruleus	SC	superior colliculus
LH	lateral hypothalamus	Str	striatum
M1	primary motor cortex	VLM	ventrolateral medulla (caudal)
MDT	medial dorsal thalamus	VPT	ventral posterior thalamus
PAG	periaqueductal gray		

1.3 Medullospinal neurons

The RVM projects to the spinal cord with axonal collaterals terminating along the rostrocaudal axis in both the spinal ventral horn and SDH [93]. It thereby acts as a relay for descending modulation of the vasomotor tone and nociception [33]. Originating from the RVM serotonergic (5-hydroxytryptamine; 5-HT) medullospinal neurons display partial topographic specificity. In particular, neurons that display co-localization of 5-HT with peptides terminate in the ventral horn and deep layers of the SDH [61,75,107]. On the other hand, neurons that display co-localization of 5-HT with GABA terminate exclusively in superficial layers of the SDH [24,107].

GABA acts as an inhibitory neurotransmitter in healthy animals, and, as evident at the supraspinal level, 5-HT neurons can express GABA receptors [170]. Therefore, GABA may calibrate the release of 5-HT from medullospinal neurons and its diffusion to adjacent neurons (i.e., volume transmission [1]) by the means of autoinhibition in the SDH. At a high level of volume transmission, all neurons can act as a 5-HT target. Depending on the receptor type they express, however, activation of a 5-HT receptor can result in either inhibition (e.g. 5-HT_1) or facilitation (e.g. 5-$HT_{2\text{-}4}$) of neuronal activity (for a review, see [41]). Furthermore, whether such modulation of neuronal activity in the SDH leads to pro- or antinociception depends on the role 5-HT target neurons play in nociception (transmission, facilitation, or inhibition).

Activation of $GABA_A$ receptors leads to an influx of chloride anions and cell hyperpolarization. The transmembrane gradient of chloride anions is then restored by the chloride exporter KCC2. In neuropathic conditions, however, a decrease in KCC2 expression leads to a lower inhibitory potency of GABA. Such loss of GABA-mediated inhibition in the SDH contributes to development of chronic pain [27], whereas in the ventral horn, this loss leads to development of spasticity. In the latter case, however, a decrease in KCC2 expression results from the loss of descending 5-HT input [18]. It is therefore likely that chronic pain develops (at least in part) for a similar reason [32,46].

In the RVM, 5-HT neurons display slow and steady discharge that fails to change in response to noxious stimulation. Such discharge is exclusive to 5-HT neurons and indicates their role in tonic modulation of pain [106]. For dynamic modulation, on the other hand, non-5-HT neurons are most likely responsible. Non-5-HT neurons respond to noxious stimulation with either transient inhibition (OFF cells) or facilitation (ON cells) of their discharge. In OFF cells, ongoing discharge positively correlates with antinociception, whereas in ON cells, ongoing discharge positively correlates with pronociception [56]. The role of non-5-HT neurons that fail to respond to noxious stimulation, however, remains unclear. Together with 5-HT

neurons, unresponsive to noxious stimulation non-5-HT neurons constitute the population of NEUTRAL cells [106].

1.4 Amygdala

1.4.1 Role of the amygdala in Pavlovian conditioning

After pairing with an unpleasant stimulus, a neutral cue can become aversive, whereas, after pairing with a pleasant stimulus, it can become appetitive. Thus, depending on the conditions, a neutral cue can acquire either a negative or a positive emotional value. Such conditioning was first demonstrated more than a century ago by Ivan Pavlov, who could induce salivation in dogs with a sound of a bell after pairing it with feeding. Furthermore, replacement of an auditory with a noxious signal produced the same effect without any signs of pain. A reward of feeding likely compensated for the unpleasantness of noxious stimulation and, in a sense, led to the dogs' comprehension of the phrase "no pain, no gain". However, noxious stimulation of a body part, which remained intact during pairing, did result in unbearable pain [108,122]. In Pavlovian conditioning, pain inhibition therefore requires a circuitry for precise localization of noxious stimulation and previous experience. Later studies revealed that this circuitry incorporates a limbic brain area, the amygdala, which can indirectly (via the RVM) regulate processing of nociception throughout the spinal cord and, thereby, modulate pain threshold at different body sites [58,59,93]. In addition, the amygdala acts as a junction of pathways for polymodal sensations and emotions. The amygdala predetermines whether collision of nociceptive and non-nociceptive signals will lead to the ability of the latter to elicit negative emotions in the future, such as fear [90]. Pain-related fear represents acute stress that can raise the pain threshold and prepare the animal for the fight-or-flight response [58,59]. In chronic stress, however, fear transitions to anxiety and susceptibility to pain increases [91]. Chronic stress and anxiety represent major complaints among neuropathic patients (e.g. [129,132]) and imply that the amygdala exerts a role in pain chronification [72,74,94,112,147].

1.4.2 Role of the amygdala in nociception

The amygdala (Fig. 3) consists of a group of nuclei, each having distinct connections with pain-modulating brain structures (for a review, see [115]). The lateral amygdaloid nucleus (LA) receives polymodal sensory information from the thalamocortical network and sends it to the basolateral amygdaloid nucleus (BLA), which, in turn, relays it to the central amygdaloid nucleus (CeA) and to the medial

prefrontal cortex (mPFC). The BLA projects to the CeA either directly or, as the mPFC, via intercalated mass of inhibitory interneurons (ITC cells) in the amygdala [71,143]. The CeA, in addition, receives nociceptive-specific information from the spinal cord via the PbN and pairs it with inhibitory inputs from ITC cells or with direct modulatory inputs from the BLA [12,102]. The outcome of such pairing is then transmitted through the PAG to the RVM [59], which, as the CeA, also receives nociceptive-specific information from the PbN [139]. The RVM compares information from the PAG with information from the PbN for regulation of nociceptive processing in the SDH [59].

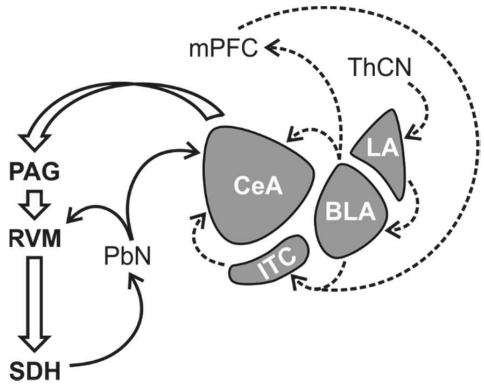

Figure 3. Role of the amygdala in nociception.

BLA	basolateral amygdaloid nucleus	**- ->**	amygdaloid nociceptive circuitry
CeA	central amygdaloid nucleus	**–>**	ascending nociceptive pathways
ITC	intercalated cell mass	**=>**	descending nociceptive pathways
LA	lateral amygdaloid nucleus		
mPFC	medial prefrontal cortex		
PAG	periaqueductal gray		
PbN	parabrachial nucleus		
RVM	rostral ventromedial medulla		
SDH	spinal dorsal horn		
ThCN	thalamocortical network		

1.4.3 Chronic pain-induced neuroplastic changes in the amygdala

Projections of the BLA to the mPFC allow pain to affect cognitive functions, whereas projections of the mPFC to amygdaloid ITC cells permit cognition to modulate pain. In chronic pain states, however, the BLA displays hyperactivity that leads to preferential excitation of inhibitory interneurons rather than of pyramidal cells in the mPFC. A decrease in capability of the mPFC to excite inhibitory ITC cells in the amygdala results in disinhibition of the CeA. Taken together, this leads to impairment of cognitive functions and pain persistence [71]. The reason for hyperactivity of amygdaloid nuclei, however, does not lie in the consistent afferent input from chronic injury. Rather, the injury leads to pain maintenance by changing the strength of amygdaloid synapses. In brain slices of animals with chronic pain, potentiation of synaptic transmission from LA to BLA [71], from BLA to CeA [44,116], as well as from PbN to CeA [65] has been shown. Brain imaging studies revealed amygdaloid hyperactivity in arthritic patients [85], whereas neuropathic patients display a decrease in amygdaloid activity as a result of long- but not short-term pain relief [47]. GABAergic inhibition of the amygdala in neuropathic animals leads to attenuation of pain hypersensitivity and affect [123], whereas stimulation with corticosterone in healthy animals leads to development of behavioral and spinal hypersensitivity [51,128]. Overall, these findings support the hypothesis that peripheral nerve injury increases amygdaloid activity, which accounts for supraspinal pain chronification.

1.4.4 Role of the amygdala in stress

1.4.4.1 General adaptation syndrome

In biology, interchange between damage and defense induces stress, which consists of three stages. The first stage features activation of the sympathetic nervous system for execution of defense behavior or, in other words, of the fight-or-flight response. The second stage involves the synthesis and release of glucocorticoids into the bloodstream to help to cope with stress. However, if stress continues, the third stage takes place, in which exhaustion, malfunction, and degradation of the body are evident. The first and second stages imply a normal general adaptation to stress agents (stressors), whereas the third stage indicates emergence of the adaptation disorder. Moreover, our susceptibility to stressors as well as their combination and specificity of action determine what type of a disorder develops [148]. In this light, neuropathic pain—a disorder in its own right—represents a result of abnormal adaptation to nerve injury. Therefore, expanding our

understanding of the mechanisms of stress maladaptations in the setting of nerve injury can provide new insights into the basis for neuropathic pain.

1.4.4.2 Hypothalamic-pituitary-adrenocortical axis

Acute stress activates sympathetic system, which enhances brain activity through elevation of sugar and oxygen blood levels. This allows rapid decision-making on how to minimize external danger. In addition, stress triggers a slower, yet a longer-lasting response characterized by a release of corticotropin-releasing factor (CRF) from the paraventricular nucleus (PVN) of the hypothalamus (for reviews, see [16,60]). CRF stimulates production of adrenocorticotropic hormone (ACTH) and beta-endorphin from pro-opiomelanocortin in the pituitary gland [103]. ACTH promotes production of glucocorticoids in the adrenal cortex, whereas beta-endorphin suppresses pain. The latter, in part, explains the analgesia evident in acute stress, during which, however, non-opioid mechanisms also take place [145].

Experimental lesions of the BLA and medial amygdaloid nucleus in rats reveal a decrease in activation of the hypothalamic-pituitary-adrenocortical (HPA) axis during acute emotional stress [13,29], whereas lesions of the CeA fail to have any effect [29,126]. On the other hand, lesions of the CeA dampen responses of the HPA axis to acute inflammatory stress [185] and late activation of the parasympathetic system in acute pain stress. Furthermore, CeA lesions abolish both early sympathetic and late parasympathetic responses to stimuli paired with acute pain stress [140]. Taken together, these studies demonstrate that amygdaloid nuclei play a pivotal role in stress regulation by initiating stress responses of the HPA axis and recovery from stress-induced sympathetic activation, as well as by forming and replaying stress memory (i.e., conditioning).

1.4.4.3 Corticotropin-releasing factor

Interestingly, the amygdala represents a major site for expression of CRF and its receptors outside the hypothalamus, suggesting a leading role of CRF circuitry in orchestrating stress and pain responses. In particular, the lateral hypothalamus (LH, through a release of CRF) and PbN (through a release of calcitonin gene-related peptide) innervate CRF-containing neurons in the CeA. In return, CRF-containing neurons in the CeA project back to the LH and PbN. It is likely that these CRF projections from the CeA regulate ascending nociceptive transmission through the LH and PbN. In addition, the CeA both directly and indirectly (through the bed nucleus of the stria terminalis) innervates CRF-containing neurons in the PVN of the

hypothalamus and, thus, likely regulates stress responses of the HPA axis (for a review, see [50]).

CRF in the amygdala acts on two G-protein-coupled receptors: an excitatory CRF_1 receptor and inhibitory CRF_2 receptor [69]. Blocking CRF_1 receptors in the CeA prevents shock-induced freezing [6], whereas its activation leads to synaptic facilitation between the CeA and parabrachial terminals and to greater neuronal output of the CeA [67]. In addition, chronic pain leads to synaptic facilitation between the CeA and the parabrachial terminals as well as between the CeA and BLA. However, blocking CRF_1 receptors can inhibit synaptic facilitation, whereas blocking CRF_2 receptors can facilitate it [45]. CRF_1 receptors require lower doses of CRF for CeA stimulation than CRF_2 receptors do for CeA inhibition [69]. Together, amygdaloid CRF_1 and CRF_2 receptors are likely to be involved in pro- and antinociceptive actions driven by the amygdala.

Interestingly, although rats with high stress reactivity display pronociception mediated by CRF_1 receptors [66], activation of CRF_1 receptors in normal rats results in pronociception, independent of the HPA axis [67]. The amygdala therefore likely regulates pain through CRF circuitry distinct from the HPA axis. Repetitive activation of the HPA axis, however, can initiate pronociceptive changes in amygdaloid expression of CRF and its receptors. This is consistent with findings showing that prolonged exposure of the CeA to glucocorticoids raises CRF expression levels in the CeA of healthy rats that leads to pain facilitation and, likely through sensitization of the HPA axis, to development of anxiety [51,149,162]. Neuropathic animals also fail to display any functional deficiencies in the HPA axis and have higher CRF expression levels in the CeA [141,166].

1.4.4.4 Reactive oxygen species

Glucocorticoids help maintain high brain activity levels by facilitating glucose availability through glycogenolysis, gluconeogenesis, and lipolysis. Moreover, glucocorticoids suppress the immune system that results in even greater availability of glucose for brain consumption (for reviews, see [125,154,155]). In chronic stress, however, suppression of immune responses and an increase in brain metabolism raise the risk of cell damage from uncontrolled ROS production (for a review, see [152]). Animal models of stress, among which are those driven by noxious stimulation, reveal a dynamic correlation between brain antioxidative activity and behavior. Initiation of antioxidative activity in the first week of stress corresponds to a decrease in exploratory behavior, which most likely indicates development of fear. In the second week of stress, antioxidative activity reaches its peak and behavior returns to normal. Beyond this period, however, antioxidative activity fails

and animals display abnormally high exploratory behavior [52], which may mask development of anxiety [156]. The amygdala therefore, as an essential brain area for development of fear and anxiety, is likely to be susceptible to oxidative changes in long-term stress. Moreover, since nerve injury can represent a form of long-term stress, similar changes in the amygdala likely take place during transition of pain from an acute to a chronic state.

Induction of hyperalgesia and comorbid anxiety with peripheral administration of pain-inducing agents is associated with an increase in amygdaloid levels of ROS, which not only promotes excitatory transmission from the PbN to the CeA but also elevates excitability and activity of the CeA [68,101]. Similarly, activation of a metabotropic glutamate receptor type 5 (mGLUR5) in the amygdala induces hyperalgesia in healthy rats by promoting ROS production and, thereby, elevating CeA activity [97]. The table below demonstrates that mGLUR5 is likely the only metabotropic glutamate receptor, which CeA neurons express on their cell bodies (Table 1). In light of this notion and the fact that the CeA acts as a sole nucleus of the amygdala that projects directly to regions, which either transmit ascending nociception or modulate it, mGLUR5 emerges as the main culprit for amygdala-driven pronociception [3,14,54,95,96,133,134].

Table 1. Glutamate receptors in the amygdala and effect of their activation.

Family	Group	Type	Synapse	Localization	CeA activity
Metabo-tropic	I	mGLUR1	ITC*→CeA	presynaptic	↑
		mGLUR5	BLA→CeA	postsynaptic	↑
	II	mGLUR2,3	ITC*→CeA	presynaptic	↓
	III	mGLUR4,7,8	PbN→CeA	presynaptic	↓
		mGLUR7	ITC*→CeA	presynaptic	↑
		mGLUR8	BLA→CeA	presynaptic	↓
Ionotropic		NMDA	PbN→CeA	postsynaptic	↑

BLA basolateral amygdaloid nucleus
CeA central amygdaloid nucleus
ITC intercalated cell mass
PbN parabrachial nucleus
* indirect evidence

1.4.4.5 Transient receptor potential channels

Transient receptor potential (TRP) channels are non-selective cation channels that are highly sensitive to cellular redox status. Increase in ROS production leads to TRP-mediated influx of cations, which can depolarize the cell membrane and act as second messengers (for a review, see [121]).

From the ankyrin subfamily of TRP channels, mammalian cells express only type 1 ($TRPA_1$), which acts as a sensor for irritant chemicals at C-fibers in the skin [77] and lungs [114]. There, activation of $TRPA_1$ channels by ROS can lead to sensitization of C-fibers [2,144] and, thus, to development of pain hypersensitivity [31]. Furthermore, in the SDH of the spinal cord, presynaptic $TRPA_1$ channels facilitate glutamatergic transmission from primary afferents [80] that likely accounts for development of secondary hypersensitivity, as evident both in patients [83] and experimental animals [177]. Similarly, presynaptic $TRPA_1$ channels facilitate glycinergic transmission onto medullary dorsal horn neurons [25]. Considering that glutamate and glycine act as co-agonists of N-methyl-D-aspartate (NMDA) channels, this suggests that activation of $TRPA_1$ channels can lead to activation of NMDA channels. Although NMDA channels in the CeA account for maintenance of neuropathic pain [3], the contribution of $TRPA_1$ channels to pronociceptive changes in the CeA remains unknown.

From the canonical subfamily of TRP channels (TRPC), the amygdala expresses type 1, 4, and 5, which unite to form two heteromeric complexes: $TRPC_1$/$TRPC_4$ and $TRPC_1$/$TRPC_5$. In the dorsal root ganglion of the sciatic nerve, expression of $TRPC_4$ channels changes bidirectionally, depending on the site of injury and the outcome (pain or regeneration) [153,183]. In the LA, activation of mGLUR1 or mGLUR5 triggers an intracellular signaling cascade that leads to activation of $TRPC_4$ and $TRPC_5$ channels responsible for mediating innate but not learned (i.e., pain-related) fear [38,135,136]. In the CeA of neuropathic animals, however, it remains unknown whether $TRPC_4$ and $TRPC_5$ channels are responsible for mediating pain hypersensitivity and pain affect.

1.5 Somatosensory cortex

As the outer layer of the brain, the cortex provides an opportunity for external modulation of brain activity with minimum invasion. Since 1991, electrical stimulation of the primary motor cortex (M1) serves as a safer and more efficacious method for attenuation of chronic pain in patients than stimulation of the thalamus [163,164]. Nowadays, TMS of the M1 provides a minimally invasive alternative to direct electrical stimulation and serves as a method for treatment of two comorbid

deceases, chronic pain [92,110] and paresis [11,163]. However, TMS of the S2 can serve as an alternative and efficient method for attenuation of neuropathic pain, when TMS of the M1 remains inefficacious [99,100,168].

The human primary (S1) and secondary (S2) somatosensory cortices receive nociceptive and non-nociceptive somatosensory information directly from the thalamus and process it in parallel [98]. The S2 displays functional topography for noxious and innocuous stimuli [39]. Furthermore, innocuous stimulation of the nearby skin can inhibit (i.e., gate) nociceptive activation of the S2, whereas noxious stimulation cannot [161]. This is consistent with the proposal by Melzack and Wall [108] that innocuous stimulation may predetermine responses of the S1/S2 to noxious stimulation and trigger S1/S2-driven modulation of the gate control system in the spinal cord (see chapter 1.2.2).

In comparison with healthy controls, patients with trigeminal neuralgia [118] or central post-stroke pain [81] display a smaller volume of the gray matter in the S2, whereas patients with chronic musculoskeletal pain show greater neural activity in the S2 during presentation of pain-related words [159]. TMS of the S2 attenuates acute pain in healthy subjects [100,168] and drug-resistant neuropathic pain in patients [99]. Importantly, in both cases, the TMS of the S2 produces greater pain attenuation than TMS of the S1 or M1. In addition, TMS of the S2 attenuates visceral pain clinically [43], but does not, however, attenuate experimental pain in interictal migraineurs [165].

In healthy rats, electrical stimulation of the S2 fails to attenuate pain during acute noxious stimulation, whereas in rats with formalin-induced pain, electrical stimulation of the S2 produces weak antinociception by itself and strong antinociception in combination with a neural NO synthase inhibitor at a subtherapeutic dose [86]. The synergism of the two treatments correlates with c-Fos expression in the spinal cord and, in part, requires activation of spinal 5-HT receptors (but not activation of opioid and noradrenergic systems) [87]. Moreover, following formalin lip injections, S2 stimulation alone can reduce c-Fos expression in dorsal-horn neurons at the medullary level, suggesting its efficacy against orofacial pain [49]. Taken together, these findings indicate that S2 stimulation is capable of attenuating pain through medullospinal 5-HT mechanisms, but only in the setting of tissue injury.

2 AIMS

The aim of the current work was to test following hypotheses:

- Neuropathic pain results in part from pronociceptive hyperactivity of the CeA. Therefore, inhibition of the CeA with intraamygdaloid injections of lidocaine should attenuate neuropathic pain in rats with peripheral nerve injury but not nociceptive pain in sham-operated rats (**IV**). Furthermore, stimulation of the CeA with intraamygdaloid injections of glutamate should exacerbate neuropathic pain (**I**).
- Oxidative changes in the CeA contribute to development of pronociceptive hyperactivity of the CeA. Therefore, intraamygdaloid injections of antioxidants should attenuate neuropathic pain in rats with peripheral nerve injury, but not nociceptive pain in sham-operated rats (**IV**). Furthermore, intraamygdaloid injections of a ROS donor should recapitulate neuropathic pain characteristics (e.g. hypersensitivity) in sham-operated rats (**IV**).
- $TRPC_{4/5}$ and $TRPA_1$ channels in the CeA act as receptors for ROS (for a review, see [121]). Therefore, intraamygdaloid injections of either a $TRPC_{4/5}$ antagonist (**II**) or a $TRPA_1$ antagonist (**IV**) should attenuate neuropathic pain in rats with peripheral nerve injury, but not nociceptive pain in sham-operated rats.

In addition, we set to identify medullospinal mechanisms for hypothesized changes in spinal nociception induced by intraamygdaloid injections (**I**, **IV**). Particularly, we attempted to prevent hypothesized changes either by inhibiting cell bodies of 5-HT neurons in the RVM (**I**) or by blocking 5-HT receptors in the spinal cord (**I**, **IV**).

We next investigated whether S2 stimulation (**III**) attenuates neuropathic pain by reversing medullospinal mechanisms that the CeA engages for descending facilitation of spinal nociception (**I**, **IV**). We assessed the effect of S2 stimulation on neuropathic and nociceptive pain and recorded the discharge rate of medullary and spinal neurons in lightly anesthetized rats. Thereafter, we assessed whether the same dugs that prevented pain attenuation induced by intraamygdaloid injections (**I**, **IV**) would prevent pain attenuation induced by S2 stimulation (**III**).

3 METHODS

3.1 Ethical considerations

All experiments (Table 2) were performed according to the guidelines of European Communities Council Directive of 22 September 2010 (2010/63/EU). The national Animal Experiment Board in Finland (ELLA) approved the methods. All efforts were made to limit distress and to use only the number of rats (RccHan:WIST, Harlan Laboratories, Netherlands) necessary to produce reliable scientific data. We followed the principles of the 3Rs (replacement, reduction, and refinement) in the design of our experiments [146].

Table 2. Allocation of methods to their corresponding studies.

		Study
Surgeries		
	SNI model	**I**, **II**, **IV**
	SNL model	**III**
	Intracranial cannula implantation	**I**, **II**, **III**, **IV**
	Intrathecal catheterization	**I**, **IV**
Behavioral tests		
	Mechanical pain	**I**, **II**, **III**, **IV**
	Heat pain	**III**
	Emotional pain	**IV**
Electrophysiology		**III**

SNI spared nerve injury
SNL spinal nerve ligation

3.2 Drugs

Glutamate (l-glutamic acid monosodium salt), 8-OH-DPAT (8-hydroxy-2-(dipropylamino)tetralin, 5-HT_{1A} receptor agonist), MK-801 (NMDA receptor antagonist), ML-204 ($TRPC_{4/5}$ channel antagonist), naloxone (opioid receptor antagonist), ondansetron (5-HT_3 receptor antagonist), PBN (phenyl-N-tert-butylnitrone, antioxidant), raclopride (dopamine D_2 receptor antagonist), t-BOOH (tert-butyl-hydroperoxide, ROS donor), TEMPOL (4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl, antioxidant), WAY-100635 (5-HT_{1A} receptor antagonist), and A-967079 ($TRPA_1$ channel antagonist) were purchased from Sigma-Aldrich (St. Louis, MO, USA). Atipamezole (α_2-adrenoceptor antagonist), lidocaine (sodium channel blocker), and pentobarbital ($GABA_A$ receptor positive allosteric modulator) were purchased from OrionPharma (Espoo, Finland). CHEMBRIDGE-5861528 ($TRPA_1$ channel antagonist) was purchased from ChemBridge (San Diego, CA, USA). Buprenorphine (μ-receptor partial agonist, κ-receptor antagonist) was purchased from RB Pharmaceuticals Limited (Slough, UK).

3.3 Surgeries

3.3.1 Anesthesia

All surgeries were performed in rats anesthetized with intraperitoneal injections of pentobarbital at the dose of 60 mg per kg. Additional injections of pentobarbital were given at the dose of 15-20 mg per kg as needed to maintain deep level of anesthesia. Following surgery, rats received subcutaneous injections of buprenorphine at the dose of 0.01-0.03 mg per kg for three consecutive days every twelve hours. Rats were allowed to recover for at least one week before the experiments (Fig. 4).

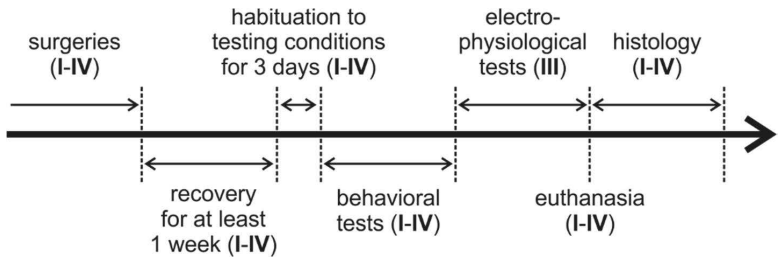

Figure 4. Time course of studies I-IV.

3.3.2 Spared nerve injury model

The spared nerve injury (SNI; Fig. 5) model of neuropathic pain derives its name from sparing the sural nerve while cutting the other two branches of the sciatic nerve (tibial and common peroneal nerves) [30]. Such partial denervation induces pain hypersensitivity in the lateral plantar surface of the injured paw for six or more months.

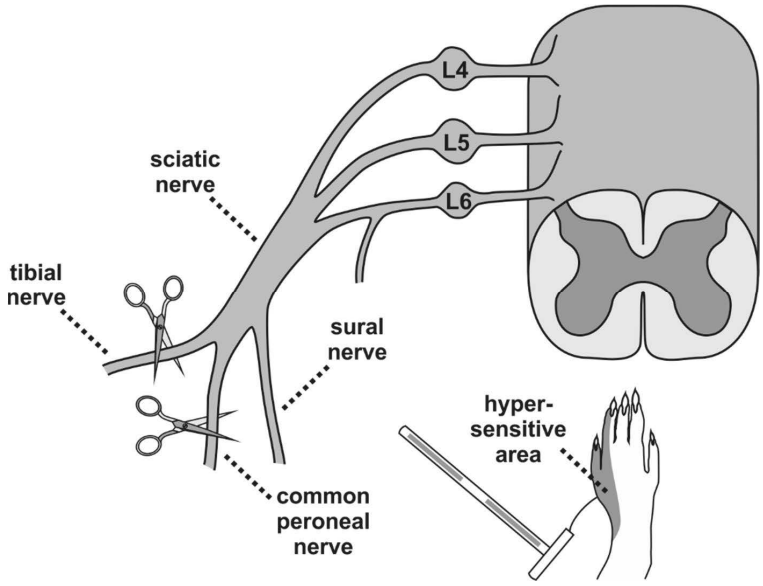

Figure 5. Spared nerve injury model.

3.3.3 Spinal nerve ligation model

Unilateral ligation of spinal nerves L5 and L6 served as a second model of neuropathic pain [78]. In contrast to SNI, rats with spinal nerve ligation (SNL; Fig. 6) develop pain hypersensitivity in the entire plantar surface of the injured paw. Hypersensitivity in the SNL model, however, lasts for a much shorter duration (up to two months).

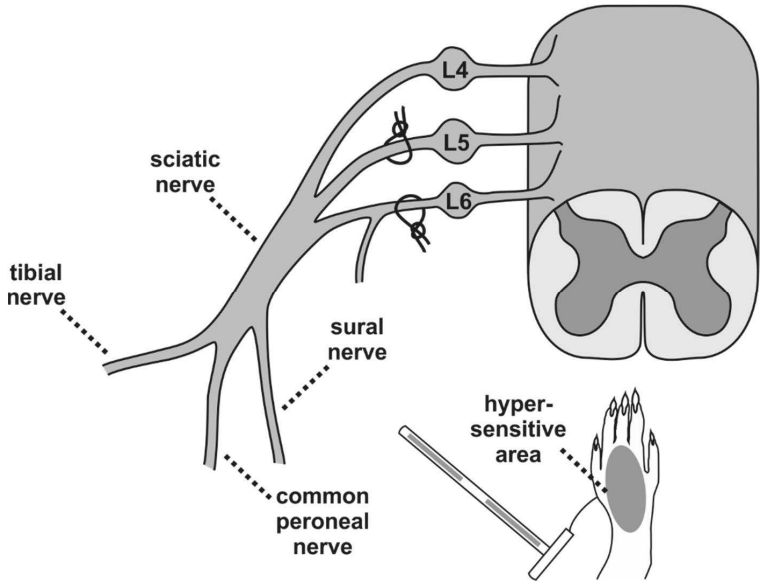

Figure 6. Spinal nerve ligation model.

3.3.4 Intracranial cannula implantation

Anesthetized rats were secured to a stereotaxic frame and a hole was made in the skull for the implantation of a chronic guide cannula 2 mm above the desired injection site. The guide cannula was cemented to screws, which, in turn, were anchored to the skull. During experiments, injections were made with the use of an internal cannula protruding 2 mm from the tip of the guide cannula. At the completion of experimental procedures, the rats were euthanized, and injection sites were identified in the coronally sectioned brain after fixation in formalin.

3.3.5 Intrathecal catheterization

Anesthetized rats were implanted with an intrathecal catheter for lumbar drug injections. The implantation was considered successful if a lidocaine injection through the catheter produced temporal paralysis of hind limbs.

3.4 Behavioral tests

3.4.1 Mechanical pain

Rate of paw withdrawal during mechanical stimulation with ascending series of calibrated von Frey filaments indicated the level of mechanical pain sensitivity.

3.4.2 Heat pain

Latency of paw withdrawal during heat stimulation with a Peltier thermode indicated the level of heat pain sensitivity.

3.4.3 Emotional pain

To evaluate the aversive component of pain, an aversive place-conditioning paradigm was used [89], in which a neuropathic rat resided in a chamber divided into two halves, one dark and the other illuminated. In the dark half, the injured paw was stimulated, whereas, in the illuminated half, the healthy paw was stimulated. As a result, the rat chose the illuminated half over the more naturally preferable dark half. A drug-induced increase in time the rat spent in the dark half indicated attenuation of emotional pain.

3.5 Electrophysiology

The discharge rate of neurons was recorded with an extracellular electrode in lightly anesthetized rats secured to a stereotaxic frame. In the RVM, neurons giving excitatory responses to noxious stimulation were considered as pronociceptive ON cells, whereas those giving inhibitory responses were considered as antinociceptive OFF cells [56]. In the SDH, neurons giving excitatory responses to both noxious and innocuous stimulation were considered as pain-relaying WDR cells.

4 RESULTS AND DISCUSSION

4.1 Medullospinal 5-HT neurons as a relay for bidirectional modulation of pain hypersensitivity by the amygdala (I, IV)

As a first step in investigating the role of the amygdala in neuropathic pain, we studied how inhibition of the right CeA with lidocaine influences pain in neuropathic and non-neuropathic conditions. We chose the right CeA as it plays a greater role in the development of inflammatory pain than the left CeA, independent of the location of inflammation [23,70]. Furthermore, the right CeA was contralateral to the nerve injury. A previous study in similar settings demonstrates greater pronociceptive changes in the right CeA than in the left one [65]. In our experiments, lidocaine injection (4 %, 0.5 µl) into the CeA attenuated pain hypersensitivity in neuropathic rats (SNI model) but not nociceptive pain in sham-operated rats (Table 3). This indicates that pain hypersensitivity in peripheral neuropathy develops, at least in part, due to pronociceptive hyperactivity of the CeA. This is consistent with other studies, which demonstrate positive correlation of amygdaloid activity with the level of chronic pain [44,47,65,71,85,116].

However, because lidocaine inhibits both neurons that originate from the CeA and neurons that pass through the CeA, our second step was to investigate how pain hypersensitivity changes during modulation of synaptic activity in the CeA with various doses of glutamate. A high dose of glutamate (100 µg) in the CeA attenuated hypersensitivity, whereas a low dose of glutamate (9 µg) facilitated it. Furthermore, blocking NMDA receptors in the CeA with MK-801 prevented facilitation of hypersensitivity induced by a low dose of glutamate but not attenuation of hypersensitivity induced by a high dose of glutamate. In these experiments, we pretreated the CeA with MK-801 at a dose that was too low to influence pain hypersensitivity by itself. However, in another set of experiments, attenuation of pain hypersensitivity was evident after the dose of MK-801 was elevated. Taken together, this suggests that, in the CeA of neuropathic animals, endogenous release of glutamate causes activation of NMDA receptors that dominates over activation of non-NMDA glutamate receptors. As a result, the CeA displays pronociceptive hyperactivity in neuropathy. This is consistent with a previous study that demonstrates contribution of NMDA receptors in the CeA to development of pain hypersensitivity [3]. On the other hand, there is also evidence that non-NMDA glutamate receptors in the CeA modulate pain hypersensitivity bidirectionally, depending on their type and site of expression [54,96,116,134]. However, it was outside the scope of this thesis to identify which non-NMDA

glutamate receptors in the CeA mediated attenuation of hypersensitivity induced by a high dose of glutamate.

To determine if CeA treatment with a high dose of glutamate attenuates hypersensitivity by exerting its action at the spinal cord level, we attempted to prevent antinociceptive action of glutamate by blocking spinal receptors, which are normally associated with antinociception (for a review, see [111]). Blocking 5-HT_{1A} receptors with WAY-100635 prevented attenuation of hypersensitivity induced by a high dose of glutamate in the CeA, whereas blocking α_2-adrenoceptors (with atipamezole), or dopamine D_2 receptors (with raclopride), or opioid receptors (with naloxone) failed to do the same. Furthermore, spinal injections by themselves had no effect on hypersensitivity at the currently used doses. Considering that 5-HT in the spinal cord originates entirely from supraspinal regions [88], this indicates that the antihypersensitivity effect induced by a high dose of glutamate in the CeA is mediated primarily by spinally projecting 5-HT neurons. This is consistent with other studies, which demonstrate the importance of spinal 5-HT_{1A} receptors in mediating attenuation of hypersensitivity in neuropathic [179] and other pathological pain states [48,186].

To determine if spinally projecting 5-HT neurons also mediate facilitation of hypersensitivity induced by a low dose of glutamate in the CeA, we attempted to prevent the pronociceptive action of glutamate by blocking the spinal 5-HT_3 receptor, which is normally associated with pronociception (for a review, see [111]). Blocking 5-HT_3 receptors with ondansetron, which alone had no effect on hypersensitivity (i.e., at the currently used dose), prevented facilitation. This indicates that spinally projecting 5-HT neurons in the medulla can relay both facilitation and attenuation of hypersensitivity from the CeA. Although this is consistent with other studies, which demonstrate contribution of spinal 5-HT_3 receptors to the development of hypersensitivity, there is also evidence of their role in mediating antihypersensitivity. Such controversy may result from the fact that activation of spinal 5-HT_3 receptors can lead to the release of spinal GABA [55], the inhibitory potency of which can disappear at certain stages of chronic pain development [26,27].

We next investigated whether medullospinal neurons act as sources of 5-HT in the spinal cord for bidirectional modulation of hypersensitivity by the CeA. We were particularly interested in the role of the RVM, which accounts for antinociception in pain-related fear mediated by the amygdala [58,59]. We therefore pretreated the RVM with a 5-HT_{1A} autoreceptor agonist (8-OH-DPAT) in an attempt to hyperpolarize cell bodies of 5-HT neurons [142]. Although RVM pretreatment with 8-OH-DPAT by itself failed to influence hypersensitivity, 8-OH-DPAT in the RVM prevented both facilitation of hypersensitivity induced by a low dose of glutamate

and attenuation of hypersensitivity induced by a high dose of glutamate in the CeA. This indicates that the CeA regulates activation of spinal 5-HT receptors through a release of 5-HT from neurons, which descend to the spinal cord from the RVM. However, it remains unknown how the CeA exerts its bidirectional action on hypersensitivity by activating the same medullospinal 5-HT neurons. It is possible that the CeA selectively activates either pronociceptive 5-HT_3 receptors or antinociceptive 5-HT_{1A} receptors by activating different subtypes of medullospinal 5-HT neurons. Alternatively, the CeA may regulate the level of net activity of medullospinal 5-HT neurons and, thereby, titrate the release of 5-HT in the spinal cord. This notion is supported by the findings that treatment of the RVM with a low dose of glutamate leads to facilitation of nociceptive pain, whereas treatment with a high dose results in attenuation [187]. Moreover, 5-HT neurons in the RVM account for both weak and strong attenuations of nociceptive pain induced by treatment of the PAG with low and high doses of morphine, respectively [17].

In conclusion, endogenous release of glutamate predominately leads to activation of NMDA receptors, which maintain pronociceptive hyperactivity of the CeA. The CeA, in turn, exerts its pronociceptive hyperactivity by preventing antinociceptive activation of medullospinal 5-HT neurons.

Table 3. Medullospinal 5-HT neurons as a relay for bidirectional modulation of pain hypersensitivity by the amygdala.

		CeA injections		
		LIDO (IV)	**GLU100 (I)**	**GLU9 (I)**
Neuropathic rats (SNI)	**Action in the CeA**	block of sodium channels	activation of non-NMDA receptors	activation of NMDA receptors
	Action in the RVM	–	activation of 5-HT cell bodies	activation of 5-HT cell bodies
	Action in the SDH	–	activation of 5-HT_{1A} receptors	activation of 5-HT_3 receptors
	Pain hyper-sensitivity	↓	↓	↑
	Pain affect	–	–	–
Sham rats	**Nociceptive pain**	0	–	–

CeA	central amygdaloid nucleus	↑	exacerbation
GLU100	100 µg of glutamate	↓	attenuation
GLU9	9 µg of glutamate	0	no effect
LIDO	lidocaine	–	not studied
RVM	rostral ventromedial medulla		
SDH	spinal dorsal horn		
SNI	spared nerve injury model		

4.2 Reactive oxygen species as a culprit for pronociceptive hyperactivity of the amygdala (II, IV)

In light of recent findings [97] that glutamate in the CeA can exacerbate nociceptive pain by promoting production of ROS, we addressed the role of ROS in the development of neuropathic pain. To test this, we first assessed how treatment of the CeA with antioxidants (TEMPOL and PBN) influences pain in neuropathic and non-neuropathic conditions. Treatment of the CeA with either of the two antioxidants attenuated pain hypersensitivity in neuropathic rats (SNI model). In sham-operated rats, however, neither TEMPOL nor PBN influenced nociceptive pain (Table 4). Thereafter, we addressed whether attenuation of pain affect accompanies attenuation of hypersensitivity in neuropathic rats after treatment of the CeA with antioxidants. We chose TEMPOL from the two antioxidants and observed that it attenuates pain affect. This suggests that neuropathy induces oxidative changes in the CeA that may account for its pronociceptive and pain-affect-inducing hyperactivity. This is consistent with other studies demonstrating contribution of ROS in the CeA to the development of visceral pain and bee venom-induced pain [68,101].

Because ROS act as endogenous agonists of TRP channels (for a review, see [121]), we next investigated if $TRPC_{4/5}$ and $TRPA_1$ channels in the CeA mediate observed oxidative changes. We therefore assessed how treatment of the CeA with antagonists of a $TRPC_{4/5}$ channel (ML-204) and of a $TRPA_1$ channel (CHEMBRIDGE-5861528) influences neuropathic pain. Similar to antioxidants, ML-204 and CHEMBRIDGE-5861528 in the CeA separately attenuated both pain hypersensitivity and pain affect in neuropathic rats but not nociceptive pain in sham-operated rats. In addition, attenuation of hypersensitivity was evident after blocking $TRPA_1$ channels with an antagonist structurally different from CHEMBRIDGE-5861528, A-967079. Taken together, this indicates that, in neuropathy, ROS account for pronociceptive and pain affect-inducing hyperactivity of the CeA by acting on $TRPC_{4/5}$ and $TRPA_1$ channels. This is somewhat contrary to other studies, in which $TRPC_{4/5}$ channels in the amygdala failed to play a role in pain-related fear [135,136]. However, this controversy can be explained by the fact that these studies investigated the role of $TRPC_{4/5}$ channels in the LA, whereas we focused on their role in the CeA. Additionally, these studies [135,136] demonstrate inability of $TRPC_{4/5}$ channels to influence pain-related fear in healthy (knockout) animals, whereas we examined the ability of $TRPC_{4/5}$ channels to influence pain affect only in neuropathic animals. Considering, however, that, in our experiments, blocking $TRPC_{4/5}$ channels failed to attenuate nociceptive pain in non-neuropathic (sham-

operated) animals, it is likely that $TRPC_{4/5}$ channels account for pain affect and pain-related fear only in the setting of nerve injury.

Since activation of 5-HT_{1A} receptors in the spinal cord accounted for attenuation of hypersensitivity induced by a high dose of glutamate in the CeA, we next investigated whether they also account for attenuation of hypersensitivity seen after blocking $TRPA_1$ channels in the CeA with CHEMBRIDGE-5861528. As expected, blocking spinal 5-HT_{1A} receptors with WAY-100635 at a dose that alone failed to influence hypersensitivity prevented the antihypersensitivity effect of CHEMBRIDGE-5861528. Considering that a high dose of glutamate in the CeA attenuates hypersensitivity by activating medullospinal 5-HT neurons, this suggests that blocking $TRPA_1$ channels in the CeA leads to a similar activation of medullospinal 5-HT neurons.

Encouraged by above findings, we attempted to recapitulate hypersensitivity in sham-operated rats by treating the CeA with a ROS donor, t-BOOH. Although, t-BOOH in a previous study exacerbated nociceptive pain after administration into the spinal cord [180], it failed to do so in the current study after administration at the same dose into the CeA. This indicates that acute administration of ROS alone is insufficient to induce pronociceptive changes in the CeA and thus suggests that its chronic administration or presence of comorbid factors (or both) is necessary.

In conclusion, ROS prevent antinociceptive activation of medullospinal 5-HT neurons by promoting pronociceptive hyperactivity of the CeA.

Table 4. Reactive oxygen species as a culprit for pronociceptive hyperactivity of the amygdala.

		CeA injections				
		TEMPOL (IV)	**PBN (IV)**	**ML-204 (II)**	**CHEM (IV)**	**t-BOOH (IV)**
Neuropathic rats (SNI)	**Action in the CeA**	anti-oxidant	anti-oxidant	block of $TRPC_{4/5}$ channels	block of $TRPA_1$ channels	ROS donor
	Action in the RVM	–	–	–	–	–
	Action in the SDH	–	–	–	activation of 5-HT_{1A} receptors	–
	Pain hyper-sensitivity	↓	↓	↓	↓	–
	Pain affect	↓	–	↓	↓	–
Sham rats	**Nociceptive pain**	0	0	0	0	0

CeA	central amygdaloid nucleus	↓	attenuation
CHEM	CHEMBRIDGE-5861528	0	no effect
PBN	phenyl-N-tert-butylnitrone	–	not studied
ROS	reactive oxygen species		
RVM	rostral ventromedial medulla		
SDH	spinal dorsal horn		
SNI	spared nerve injury model		
t-BOOH	tert-butyl-hydroperoxide		
TEMPOL	4-hydroxy-2,2,6,6-tetramethylpiperidine-1-oxyl		

4.3 Medullospinal 5-HT neurons as a relay for attenuation of neuropathic pain by cortical stimulation (III)

TMS of the S2 has proven to be an efficacious method for attenuation of drug-resistant pain in neuropathic patients [99]. However, because the mechanism of pain attenuation remains largely unknown, we examined whether descending pathways, which are activated by S2 stimulation, overlap with those pathways that are activated by a high dose of glutamate in the CeA. We therefore investigated whether medullary 5-HT or non-5-HT neurons contribute to S2 stimulation-induced pain attenuation in neuropathic rats. To test this, we recorded neurons in the RVM in parallel with behavioral assessment of pain. In addition, we separately recorded pain-relay neurons in the SDH. However, because SDH recordings required irrecoverable surgical preparations (laminectomy), we performed them in lightly anesthetized rats. Furthermore, to obtain comparable data, assessment of pain behavior and simultaneous recordings of medullospinal neurons were also performed in lightly anesthetized rats. However, because light anesthesia diminishes the withdrawal reflex, we used a piezoelectric movement detector over the gastrocnemius muscle to assess the reflex mechanomyographically. Furthermore, we replaced noxious mechanical stimulation with noxious heat stimulation to eliminate artifacts in the mechanomyogram. This, in turn, required implementation of the SNL model of neuropathic pain instead of the SNI model to enlarge the hypersensitive surface of the paw at the expense of the duration of hypersensitivity: two months in SNL rats versus six (or more) months in SNI rats [30,78].

Stimulation of the S2, independent of the hemisphere, attenuated heat pain in nerve-injured rats with mechanical hypersensitivity. Attenuation of heat pain was evident at the lowest cortical stimulus intensity of 30 µA (0.1-ms pulses at 300 Hz). The antinociceptive effect failed to increase at higher intensities of 50 and 70 µA. This suggests that pain attenuation was induced by stimulation of the S2 and not by a possible spread of electrical current to adjacent regions. Stimulation of the right S2 (contralateral to the injury), however, failed to attenuate heat pain in sham-operated rats and in rats that failed to develop mechanical hypersensitivity after SNL (Table 5). Although it remains unknown whether S2 stimulation in the left hemisphere attenuates pain in sham-operated and non-hypersensitive neuropathic rats, this suggests that S2 stimulation, at least in the right hemisphere, is efficacious only in animals with pathological pain hypersensitivity. This is consistent with previous findings that S2 stimulation attenuates inflammatory but not nociceptive pain [64,86].

Thereafter, we performed recordings in the SDH during stimulation of the right S2 to investigate spinal mechanisms for behavioral attenuation of heat pain in hypersensitive neuropathic rats. In particular, we recorded wide-dynamic range (WDR) cells, which accelerated their discharge rate in response to both noxious heat stimulation and innocuous mechanical stimulation of the injured paw. We observed that S2 stimulation attenuates heat responses of WDR cells (Fig. 7). We observed also that this attenuation can be prevented by blocking spinal 5-HT_{1A} receptors with WAY-100635 at a dose that alone failed to reduce heat-response discharge. Considering that, at the current dose, WAY-100635 also failed to reduce the ongoing discharge rate of WDR cells, this suggests that S2 stimulation attenuated pain by activating 5-HT_{1A} receptors, which are presynaptic to WDR cells. However, one must be cautious with this interpretation, since failure of WAY-100635 to reduce the ongoing discharge rate of WDR cells may have resulted from the fact that the ongoing discharge rate of WDR cells was originally very low. On the other hand, a recent whole-cell patch clamp study in spinal cord slices supports our interpretation of presynaptic localization of 5-HT_{1A} receptors, by demonstrating that 5-HT in the SDH inhibits nociceptive transmission via activation of 5-HT_{1A} receptors, which are localized on spinal terminals of primary afferents [160].

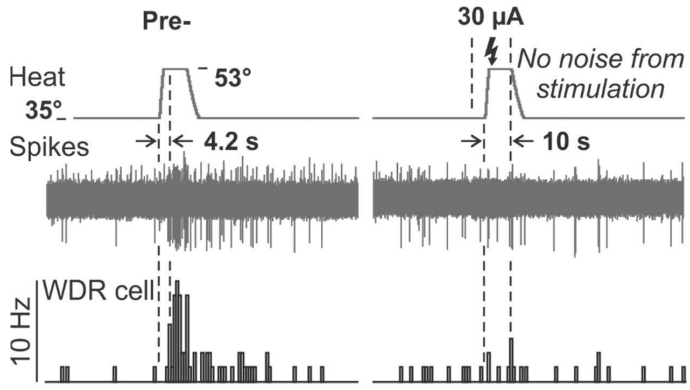

Figure 7. Example of recordings in the spinal dorsal horn.

Because both electrical stimulation of the S2 and glutamatergic stimulation of the CeA attenuate pain in hypersensitive neuropathic rats by activating spinal 5-HT_{1A} receptors, it is likely that they do so through the same mechanism. To test this hypothesis, we attempted to prevent S2 stimulation-induced pain attenuation by pretreating the RVM with an agonist of 5-HT_{1A} autoreceptors (8-OH-DPAT), which prevented pain attenuation induced by a high dose of glutamate in the CeA. In the S2 experiments, as in the CeA experiments, we used 8-OH-DPAT at a dose, which failed to influence pain by itself. In support of our hypothesis, 8-OH-DPAT prevented S2 stimulation-induced pain attenuation. As in the CeA experiments, we presume that 8-OH-DPAT acted by inhibiting cell bodies of spinally projecting 5-HT neurons. This indicates that activation of medullospinal 5-HT neurons is a necessary step for the S2 stimulation-induced pain attenuation, as it is for pain attenuation induced by a high dose of glutamate in the CeA. However, it remains unknown whether S2 stimulation and a high dose of glutamate in the CeA attenuate pain by recruiting the same subtypes of medullospinal 5-HT neurons or by regulating their net activity in a similar fashion.

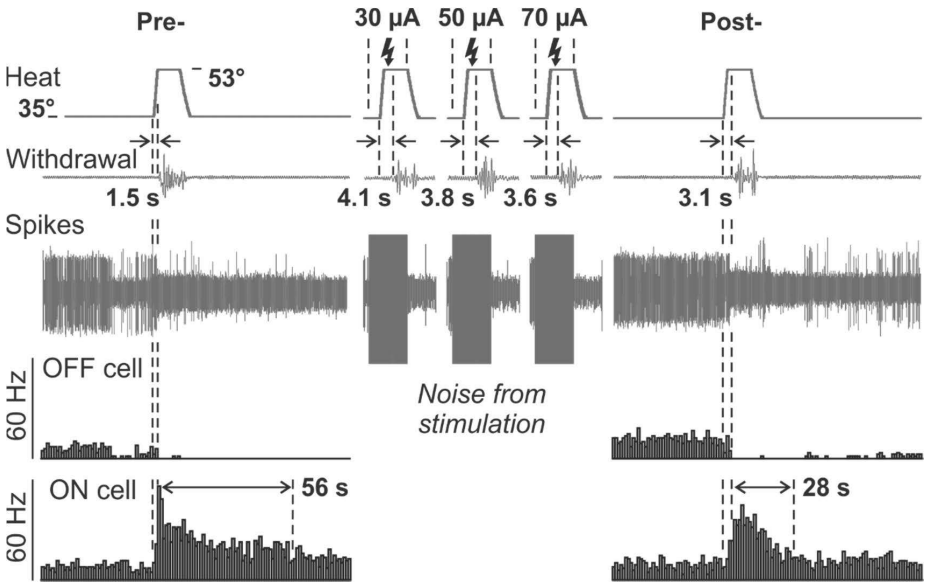

Figure 8. Example of recordings in the rostral ventromedial medulla and parallel assessment of pain behavior.

In addition, we recorded heat responses of pain-regulating ON and OFF cells in the RVM, which are shown to be non-5-HT [106]. Unlike WDR cells in the SDH, which respond to both noxious and innocuous stimulations, ON and OFF cells in the RVM respond only to noxious stimulation. Furthermore, ON cells respond by accelerating their discharge rate before withdrawal of the paw, whereas OFF cells respond by decelerating it. A fast discharge rate of ON cells and a slow discharge rate of OFF cells predict facilitation of nociception, whereas a slow discharge rate of ON cells and a fast discharge rate of OFF cells predict inhibition of nociception [56]. However, in our experimental setup, we were unable to record ON and OFF cells during S2 stimulation, because the cortical stimulation electrode induced stimulus artifacts, which masked all neural spikes during stimulation. Therefore, we were able to record ON and OFF cells only after cessation of S2 stimulation when attenuation of pain behavior was marginal. Nonetheless, we observed that S2 stimulation shortens duration of heat responses in ON cells and prolongs the latency of heat responses in OFF cells (Fig. 8). Considering that at least some of the ON and OFF cells project to the SDH [40,169], this suggests that S2 stimulation promotes antinociceptive activity of both non-5-HT (i.e., ON and OFF cells) and 5-HT medullospinal neurons. Therefore, our findings are consistent with a previous study [87], where unselective inhibition of spinal 5-HT receptors prevented pain attenuation induced by S2 stimulation. Interestingly, the same study [87] demonstrates also the insignificance of the opioid and noradrenergic systems in S2 stimulation-induced pain attenuation, which narrows the list of candidates for the role of (presumably medullospinal) non-5-HT neurons engaged by S2 stimulation in our study.

In conclusion, S2 stimulation represents a clinically relevant method that has the potential to recover antinociceptive activity of medullospinal 5-HT neurons, which is suppressed in neuropathy due to pronociceptive hyperactivity of the CeA.

Table 5. Medullospinal 5-HT neurons as a relay for attenuation of neuropathic pain by cortical stimulation.

		S2 stimulation (III)
Neuropathic rats (SNL)	**Action in the CeA**	–
	Action in the RVM	activation of 5-HT cell bodies
		inhibition of ON cell
		activation of OFF cells
	Action in the SDH	inhibition of WDR cells via activation of 5-HT_{1A} receptors
	Pain hyper-sensitivity	↓
	Pain affect	–
Sham rats	**Nociceptive pain**	0

CeA	central amygdaloid nucleus	↓	attenuation
RVM	rostral ventromedial medulla	0	no effect
SDH	spinal dorsal horn	–	not studied
SNL	spinal nerve ligation model		
S2	secondary somatosensory cortex		
WDR	wide-dynamic range		

4.4 General discussion

The present studies suggest that, in neuropathic conditions, endogenous release of glutamate in the CeA predominantly activates NMDA (rather than non-NMDA) receptors, which maintain pronociceptive hyperactivity of the CeA (**I**). However, activation of non-NMDA glutamate receptors in the CeA can lead to attenuation of pain hypersensitivity, which is likely due to suppression of pronociceptive hyperactivity of the CeA. CeA-induced pronociception results from activation of spinal 5-HT_3 receptors, whereas CeA-induced antinociception results from activation of spinal 5-HT_{1A} receptors. Interestingly, activation of both spinal 5-HT_3 and 5-HT_{1A} receptors requires activation of medullospinal 5-HT neurons. This indicates that the CeA normally promotes antinociceptive activity of medullospinal 5-HT neurons, whereas, in neuropathy, the CeA starts to promote their pronociceptive activity. It is likely that medullospinal 5-HT neurons have two distinct subtypes, one of which relays antinociception, whereas the other accounts for pronociception, similar to medullospinal non-5-HT neurons (antinociceptive OFF cells and pronociceptive ON cells) [40,56,106,169]. Alternatively or additionally, the level of net activity of medullospinal 5-HT neurons may determine the final effect on nociception.

We also provide evidence that ROS in the CeA can promote pronociceptive hyperactivity of the CeA through activation of $TRPA_1$ and $TRPC_{4/5}$ channels (**II**, **IV**). In light of these observations, an increase in ROS production and a decrease in ROS detoxification in the CeA emerge as possible maladaptive changes that account for the development of neuropathic pain. Furthermore, considering that ROS represent a product of mitochondrial activity (for a review, see [113]), neuropathic pain can in part represent a mitochondrial disorder. This is consistent with previous findings that chemotherapy induces neuropathic pain by promoting accumulation of atypical mitochondria in peripheral nerves, which is associated with ROS overproduction and poor ROS detoxification [8,184].

In addition, we established that S2 stimulation attenuates neuropathic pain by inhibiting spinal transmission of nociceptive information through WDR cells (**III**). To accomplish this, S2 stimulation employs medullospinal 5-HT neurons to activate spinal 5-HT_{1A} receptors, which are likely presynaptic to WDR cells. Interestingly, such mechanism for pain attenuation in SNL rats is similar to the one that the CeA engages in SNI rats. This indicates that malfunction of medullospinal 5-HT neurons may be an important contributor to the development of chronic pain in peripheral neuropathies. Furthermore, this suggests that S2 stimulation acts by reversing CeA-driven pronociceptive activation of medullospinal 5-HT neurons. Other mechanisms for S2 stimulation-induced pain attenuation include antinociceptive changes in

activity of (presumably medullospinal) non-5-HT neurons (ON and OFF cells) [40,56,106,169].

5 CAVEATS

Although intracranial cannulae targeted the CeA (**I**, **II**, **IV**), the volume of injections was large enough (0.5 µl) for drugs to spread to adjacent brain structures, including other amygdaloid nuclei. Therefore, the action of drugs on neurons outside the CeA may have interfered with our results. On the other hand, the CeA acts as the output nucleus of the amygdala with regard to pain modulation. Furthermore, injection of drugs into a control site (internal capsule) failed to produce any alterations in pain behavior. Taken together, this indicates that changes in pain behavior, most likely, resulted from the action of drugs on neurons in the CeA.

Caution is necessary in comparing the results collected from the different studies in this thesis. In amygdaloid studies (**I**, **II**, **IV**), we assessed mechanical pain in freely moving SNI rats. In the cortical study (**III**), we assessed heat pain in lightly anesthetized SNL rats. Such inconsistency in experimental settings may have introduced bias to our results, which demonstrate overlap in mechanisms for pain regulation by the amygdala (i.e., CeA) and the cortex (i.e., S2). Furthermore, not all aspects of pain were assessed. In studies **II** and **IV**, we assessed sensory-discriminative and affective-motivational aspects of pain, whereas in studies **I** and **III**, we assessed only the former. We also did not assess ongoing pain in any of the studies, although ongoing pain represents a major complaint among neuropathic patients.

We inhibited medullospinal 5-HT neurons by activating 5-HT_{1A} autoreceptors on their cell bodies with 8-OH-DPAT injections into the RVM (**I**, **III**). We, however, cannot exclude that activation of 5-HT_{1A} heteroreceptors on other cells (neural [142] or glial [5]) influenced our results.

Although previous studies demonstrate that at least some of the ON and OFF cells in the RVM have spinal projections [40,169], we did not verify that the ON and OFF cells recorded in our study (**III**) indeed represented medullospinal neurons. Furthermore, the RVM can comprise ascending projections from the area around the central canal of the spinal cord [62]. If these ascending projections contribute to transmission of nociception, then they may resemble electrophysiologically ON or OFF cells. Collision testing, for example, could have clarified whether recorded in our study (**III**) RVM cells were ascending or descending.

6 FUTURE PROSPECTS

We demonstrated that pain-modulating pathways descending from the S2 (**III**) and the CeA (**I**, **IV**) merge in the RVM. It is therefore possible that the S2 and the CeA modulate pain in tandem. Future studies may address whether S2 stimulation attenuates neuropathic pain by modulating the activity of the CeA or by modulating the activity of the PAG, which connects the CeA to the RVM.

We also demonstrated that administration of antioxidants or $TRPA_1$ antagonists into the CeA attenuates neuropathic pain (**IV**), which is consistent with previous findings of their antinociceptive properties in the spinal cord [178,180]. From a clinical perspective, it is therefore of great interest to see if antioxidants and $TRPA_1$ antagonists can attenuate neuropathic pain, when administered systemically, and to examine whether their action at the spinal or supraspinal level plays a more important role in attenuation of neuropathic pain. In addition, it is interesting to see if systemic administration of antioxidants can potentiate antinociceptive action of S2 stimulation.

A recent clinical study [99] demonstrated that the S2 is an effective cortical stimulation site for attenuating neuropathic pain with TMS. In line with this, our study (**III**) shows that electrical stimulation of the S2 also attenuates neuropathic pain in rats and reveals the mechanism for S2 stimulation-induced pain attenuation. It remains to be studied whether a similar mechanism contribute to pain attenuation induced by TMS of the S2 in neuropathic patients.

Implementation of antidepressants, such as selective serotonin reuptake inhibitors (SSRI), has produced controversial results in treatment of neuropathic pain (for a review, see [82]). This is consistent with our findings, which suggest that medullospinal 5-HT neurons can account for both attenuation (**I**, **III**, **IV**) and exacerbation (**I**) of neuropathic pain. Investigation of how various SSRI influence the activity of medullospinal 5-HT neurons may lead to improved clinical use.

Since electrical stimulation of the S2 (**III**) and pharmacological inhibition of the CeA (**I**, **IV**) attenuated neuropathic pain by activating spinal 5-HT_{1A} receptors, it may be expected that spinal administration of a 5-HT_{1A} agonist also attenuates neuropathic pain. However, since pain attenuation induced by electrical stimulation of the S2 and by pharmacological inhibition of the CeA can be prevented by activation of 5-HT_{1A} receptors in the RVM (**I**, **III**), systemic administration of a 5-HT_{1A} agonist may prove inefficacious. In light of this notion, spinal (but not systemic) administration of BUSPIRONE may serve as a new method for clinical treatment of neuropathic pain. BUSPIRONE represents one of the few clinically approved drugs that acts as a 5-HT_{1A} receptor agonist. Currently, BUSPIRONE is used to treat anxiety but not

neuropathic pain, despite the fact that anxiety represents one of the comorbidities of neuropathic pain. The reason for this lies in inefficacy of BUSPIRONE to attenuate pain in neuropathic patients when administered systemically [79]. Our studies (**I**, **III**, **IV**), however, raise the hypothesis that spinal administration of BUSPIRONE may prove efficacious.

7 CONCLUSIONS

In neuropathic conditions:

- NMDA receptors in the CeA maintain its pronociceptive hyperactivity characterized by activation of spinal 5-HT_3 receptors (**I**).
- Activation of non-NMDA glutamate receptors in the CeA suppresses its pronociceptive hyperactivity and leads to descending antinociception mediated by spinal 5-HT_{1A} receptors (**I**).
- ROS contribute to CeA-driven pain hypersensitivity and pain affect through activation of $TRPA_1$ and $TRPC_{4/5}$ channels in the CeA (**II**, **IV**).
- Activation of medullospinal 5-HT neurons is necessary for both CeA-induced facilitation and inhibition of spinal nociception (**I**, **IV**).
- S2 stimulation attenuates neuropathic pain by promoting antinociceptive activity of medullospinal 5-HT neurons and of (presumably medullospinal) non-5-HT neurons (ON and OFF cells; **III**).

By summarizing the results of the present studies (**I**-**IV**), the table below (Table 6) describes the mechanisms of neuropathic pain development and suggests new approaches to its treatment.

Table 6. Mechanisms of neuropathic pain development and possible approaches to its treatment.

		RVM	SDH	Pain
CeA	activation of NMDA receptors	pronociceptive activation of medullospinal 5-HT neurons	activation of 5-HT_3 receptors	↑
	activation of $TRPA_1$ & $TRPC_{4/5}$ receptors with ROS			
CeA	block of NMDA receptors with antagonists	antinociceptive activation of medullospinal 5-HT neurons	activation of 5-HT_{1A} receptors	↓
	activation of non-NMDA glutamate receptors			
	block of $TRPA_1$ & $TRPC_{4/5}$ receptors with antagonists			
	detoxification of ROS with antioxidants			
S2	electrical stimulation	antinociceptive activation of medullospinal 5-HT neurons	activation of 5-HT_{1A} receptors	↓
			inhibition of WDR cells	
		antinociceptive activation of (likely medullospinal) non-5-HT neurons (ON and OFF cells)	–	

CeA	central amygdaloid nucleus	↑	exacerbation
ROS	reactive oxygen species	↓	attenuation
RVM	rostral ventromedial medulla	–	not studied
SDH	spinal dorsal horn		
S2	secondary somatosensory cortex		
WDR	wide-dynamic range		

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